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(71) Applicant *(for all designated States except US)*:
TONEN CORPORATION (JP/JP)
1-39 Hiroo 1-chome, Shibuya-ku, Tokyo 150-8411 (JP)
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*:
AOYAGI, Katsumi (JP/JP)
OHUE, Chiharu (JP/JP)
IIDA, Kumiko (JP/JP)
YAGI, Shintaro (JP/JP)
c/o Tonen Corporation General Laboratory, 3-1 Nishi Tsurugaoka 1-chome, Oi-machi, Iruma-gun, Saitama-ken 356-8505 (JP)
(74) Agent:
ISHIDA, Takashi et al.
Seiwa Patent Law Offices, Mori Bldg., 5-1-37 Toranomon, Toranomon 3-chome, Minato-ku, Tokyo 105-8423 (JP)

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(54) **Title:** METHOD FOR ASSAYING HEPATITIS C VIRUS

(57) **Abstract:**

A method for assaying hepatitis C virus (HCV) distinguished in that assaying is carried out based on binding of HCV core antigen and HCV core antibody to probes thereof in the presence of a cationic surfactant and/or a nonionic surfactant.

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Specification

Method for assaying hepatitis C virus

Field of the invention

The present invention relates to methods for detecting hepatitis C virus (HCV), and more specifically, to methods for assaying HCV core antigen or simultaneously assaying HCV core antigen and HCV core antibodies. This method is particularly effective for screening of large numbers of blood samples and the like.

Technical background

Hepatitis caused by HCV (hepatitis C virus) infection becomes chronic at a high frequency, with the prolonged infection period often leading to cirrhosis of the liver and liver cancer. However, since HCV infection is brought in primarily through blood and blood-derived components, it is possible to block off the route of infection by identifying and eliminating the infection source. Currently, methods of identifying infection sources mainly involve detecting HCV polypeptide antibodies, but methods capable of more accurately identifying infection sources are being sought.

The background of what is being sought lies in the fact that after HCV infection, there is a period in which antigen is present but antibodies are not produced: the so-called window period. In this period, blood serum cannot be judged to be infected or not by means of antibody tests. With antibody tests, because it is not possible to eliminate specimens which are in the window period, when using blood transfusions and blood-derived substances such as blood components and blood preparations screened by means of antibody tests, there is a risk of secondary infection due to specimens which are in the window period. Because of this, it was necessary to detect not HCV polypeptide antibodies, but rather HCV itself, i.e. HCV particles.

Detecting and assaying HCV itself is possible through detection of antigens or genes (RNA) making up HCV particles. Here, the antigens making up HCV particles are thought to be core antigen and envelope antigens (E1, E2).

Of these, the envelope antigens have much variation in high-antigenicity regions, as typified by the hyper-variable region. Furthermore, differences between genotypes are

also reported. In order to detect all these variations and differences, it is necessary to use probes which bind specifically with multiple regions.

Here, probes refer to molecules which bind specifically to antigens, for instance receptors, antibodies, recombinant antibodies, functional molecules or functional structures which recognize and bind to antigen molecules.

Now, in core antigens, the sequence is preserved at the amino acid sequence level, and by selecting the region, it is possible to obtain probes capable of detecting antigens of any HCV genotype for which multiple genotypes are reported, making it possible to develop detection methods which are not genotype-dependent.

However, there is a point which must be borne in mind in developing systems for detecting antigens. Namely, there is a high possibility of antibodies being present in the specimen tested, which antibodies compete for binding sites with the probe used to detect the antigen, inhibiting the probe's binding and thus lowering the antigen detection sensitivity. Thus, in order to efficiently detect antigens, methods may be considered which use probes that recognize regions to which antibodies do not bind or where antibody binding does not interfere with probe binding. However, for molecules such as HCV core antigen, for which multiple antibody binding sites have been reported, it is difficult to prepare a probe which meets the above conditions.

Thus, in order to detect the antigen molecules, it is necessary to eliminate antibodies which inhibit the probe's binding. Methods of elimination include removal based on physical principles, for instance methods of separating and sorting HCV particles and antibodies by making use of differences in molecular weight. Examples of this include gel filtration, ultracentrifuging, density gradient centrifuging, and molecular weight fractionation using membranes such as ultrafilter membranes. However, since antibodies often form complexes with other biopolymers and vary in macromolecular weight, with methods based on physical principles, sorting of HCV particles becomes difficult. Furthermore, these methods employ specialized processing equipment and the like, making difficult their application to mass screening, such as blood screening.

There are also methods based on biochemical principles, such as varying the aqueous environment of PEG (polyethylene glycol) or the like in order to sort HCV particles by preferential precipitation making use of differences in chemical properties between HCV

particles and other serum components, for instance differences in water solubility, but antibodies or antibody complexes often precipitate in the same fraction as the particles, so the fractionation itself is problematic. Furthermore, HCV particles often form immune complexes of antigens making up the HCV particle and antibodies which recognize them, and separating only the antibodies or antigens from the immune complex is difficult.

For these reasons, methods are used whereby substances (antibodies, etc.) which inhibit the probe's function are eliminated by functionally destroying them. Possible methods of causing antibodies to lose their function include modifying antibody proteins by exposing them to conditions which modify proteins, but an important point here is the condition that the antibody's function must be lost without losing the function of the target antigen, i.e. the function of binding with the probe, which, if the probe is an antibody, means not causing it to lose epitopes, or else causing it to re-exhibit epitopes.

The function required of a method for discerning the presence of HCV infection differs depending on its purpose.

Antibody tests are a method of discerning whether or not antibodies against HCV are present in a sample, but when antibodies against HCV are present in a sample, it may be the case that the sample's donor is currently infected with HCV and HCV is present in the sample, or it may be the case that HCV has already been eliminated from the organism through treatment or natural healing: it is difficult to distinguish these cases based on the presence or absence of antibodies.

With antigen tests, an important function is to discern whether HCV is present in a sample, and if it is present, what its quantity is; here, the presence or absence of antibodies does not come into question.

For treatment, in order to determine whether or not HCV is the primary cause of the hepatitis, HCV antibody tests provide important information, but ultimately, the presence or absence of HCV antigen must be determined for reliable diagnosis. Furthermore, to evaluate the efficacy of treatment, it is important to determine whether or not HCV has been eliminated from the organism, and for this determination it is important to know the quantity of antigen. That is, knowing the presence or absence of antibodies and their quantity irregardless of antibodies is important for treatment. Namely, for treatment, test

methods which give the presence or absence and the quantity of antigen are most important.

On the other hand, with blood or blood-derived preparations, preventing secondary infection is most important, for which purpose the test method must be able to discern the presence or absence of risk as an HCV infection source. Currently, antibody tests are used as the primary test method in this field.

However, as discussed above, blood serum which is in the window period following HCV infection cannot be evaluated for the presence or absence of infection by means of antibody tests. Thus, when using blood transfusions and blood-derived substances such as blood components or blood preparations which have been screened by means of antibody tests, there is a risk of secondary infection by samples in the window period.

Reducing the risk requires the concomitant use of antigen tests, but antigen tests are still not being carried out for mass screening, such as in blood tests of donor blood.

Theoretically, if there were a testing method capable of determining the presence or absence of antigen with 100% precision (sensitivity, specificity), it would suffice to use it as the sole testing method, but any detection method has its detection sensitivity, and something below the detection sensitivity cannot be determined. Thus, there are no test methods capable of determination with 100% precision. Furthermore, in specific instances, there is the possibility of infection sources being overlooked when only antigen tests are used, and thus in this field, assaying both antibodies and antigens is necessary to reduce the risk of secondary infection. The use of an antigen detection method that exhibits high sensitivity and specificity and can be applied to mass screening would require the assaying of both antigens and antibodies, increasing the number of tests for the same number of samples from what it is currently and becoming a cost increase factor.

From this it can be seen that the ability to assay antigens and antibodies with the same method would allow the number of tests to be reduced in the field in question, which would provide substantial benefits.

As was already indicated, while methods of detecting antibodies and methods of detecting antigens have been developed, when one attempts to detect antigens under the conditions for detecting antibodies as described above, the presence of antibodies which

inhibit the binding of the probe that detects antigens makes effective detection of antigens impossible. Moreover, under conditions for detecting antigens, as discussed above, the use of methods which eliminate antibodies that competitively inhibit antigen detection makes it impossible to detect the antibodies. Thus, with previously reported methods, antigens and antibodies cannot be detected with the same method.

Disclosure of the invention

For the purpose of reducing secondary infection when using blood and blood-derived substances, there is no need to distinguish infected persons and persons with a history of infection, it sufficing to be able to discern whether or not antibodies or antigens are present. Namely, the present invention provides a method whereby, for samples such as those in the window period where antibodies are not present, antigens are detected, and for samples in a period where antibodies are present, either antigens or both antigens and antibodies are detected, thereby providing a new test method as sought for the testing blood and blood-derived substances.

To solve the aforementioned problem, the present invention provides a method for assaying hepatitis C virus (HCV) distinguished in that assaying is carried out based on binding of HCV core antigen to its probe in the presence of a surfactant having an alkyl group and a secondary to quaternary amine and/or a nonionic surfactant.

The present invention furthermore provides a method distinguished in that HCV core antibody is assayed based on the binding thereof to its probe, along with assaying HCV core antigen by the aforementioned method.

Brief description of the drawings

Figure 1 is a graph comparing the antibody titer of monoclonal antibody C11-15 with the antibody titers of other monoclonal antibodies C11-3, C11-7, C11-10 and C11-14.

Figure 2 is a graph showing the results of ELISA using various inventive monoclonal antibodies singly or mixed and fixed in solid phase as the primary antibody to assay HCV-RNA positive samples.

Manner of embodiment of the invention

The HCV infection detection method provided by the present invention is a method whereby antigens are detected for samples in a period where antibodies are not present, such as the window period, and where antigens or both antigens and antibodies are detected for samples in a period where antibodies are present. Namely, since antibodies are not present in the window period, which is a period where antibodies a not present, there is no need to eliminate antibodies when detecting antigens. Thus, there is no need to carry out the pre-processing required for detecting antigens.

However, in order to detect antigens contained in HCV particles, it is important to expose the recognition sites of the probe used for detection. In an HCV virus particle, the particle is formed through the formation of a complex by nucleic acid, which is the genome, and core antigen, which particle is thought to have a structure enclosed in an outer membrane comprising a lipid membrane and envelope proteins. Particles are furthermore thought to be present in the blood as complexes formed by low density lipoprotein (LDL) with HCV antibodies and the like. Thus, with virus particles as they are found in blood, the probe cannot recognize and bind to core antigen. Therefore, in order to detect core antigen, it is necessary to carry out processing to remove these structures enclosing the core antigen, etc., so as to make the core antigen recognizable by the probe.

Namely, the present invention also provides a reaction method comprising the reaction conditions and reaction system for exposing core antigen within HCV particles contained in a specimen so as to make it recognizable to the probe used for recognition of the core antigen, and provides also the reagents comprising the reaction system.

Now, in the period when antibodies are adequately present, as described above, there are cases where antibodies against core antigen are present which compete for probe binding sites in the sample, in which case there is the possibility of a decline in core antigen detection sensitivity. Moreover, when core antigen is exposed so as to make it capable of binding with the probe, if antibodies against core antigen are present which compete with the probe, with a method which detects antibodies by detecting immune complexes, the antibodies are absorbed by the exposed core antigen and the quantity of

antibodies against core antigen which bind with the antigen used for detection decreases, so there is a possibility of a decrease in detection sensitivity.

For this reason, while the antigen used for detecting antibodies can consist solely of core antigen epitopes, it preferably comprises peptides or polypeptides which include HCV epitopes other than core antigen. Or it may be a peptide or polypeptide, or a compound, other than peptides or polypeptides containing HCV epitopes, and which mimics HCV epitopes.

However, it is preferable that the probe for detecting core antigen and the HCV epitopes or compounds substituting for HCV epitopes not be substances which bind through mutual recognition.

Antibodies used as a probe for HCV core antigen or labeled antibodies for detecting HCV core antigen may include: polyclonal antibodies obtained by immunizing experimental animals such as mice, rabbits, chickens, goats, sheep, cows, etc.; monoclonal antibodies produced by hybridomas obtained by separating spleen cells from an immunized organism and fusing them with myeloma cells; monoclonal antibodies produced by cells prepared by immortalizing spleen cells or blood leukocytes with EB virus; monoclonal antibodies produced by humans, chimpanzees, etc. infected with HCV; recombinant antibodies produced by cells prepared through transformation, by means of recombinant genes formed by combining immunoglobulin constant region gene fragments with variable region gene fragments obtained from mouse, human, etc. immunoglobulin cDNA or chromosomal DNA, variable region gene fragments formed by combining a portion of immunoglobulin cDNA or chromosomal DNA with an artificially created sequence, variable region gene fragments formed using an artificial gene sequence, or variable region gene fragments prepared by genetic recombination methods using the preceding variable region gene fragments as raw materials; phage antibodies created by fusing the aforementioned variable region gene fragments with for instance bacteriophage structural proteins; recombinant antibodies produced by cells transformed by means of recombinant antibody genes formed by combining the aforementioned variable region gene fragments with other suitable gene fragments, for instance a portion of myc gene.

Probes produced by artificially introducing variable regions into trypsin molecules, probes obtained by artificially modifying molecules which bind specifically with receptor or other proteins, probes prepared by means of other combinatorial chemistry techniques, or other molecules exhibiting a high specificity and affinity for core antigen can be used.

The aforementioned monoclonal antibodies can be easily prepared by persons skilled in the art. The preparation of monoclonal antibodies using hybridomas is well known. For instance, BALB/c mice or the like can be periodically immunized intraperitoneally or subcutaneously with the aforementioned fused polypeptide or polypeptide (hereinafter, the present antigen), singly or as an antigen bound with BSA, KLH or the like, in pure form or mixed with an adjuvant such as Freund's complete adjuvant. Once the blood antibody titer has increased, the present antigen is administered into the tail vein as a follow up immunization, the spleen is extirpated in an aseptic manner, and hybridomas are obtained by cell fusion with a suitable mouse bone marrow cell strain. This method can be carried out as per the method of Kohler and Milstein (Nature 256: 495-497, 1975).

The hybridomas cell strains obtained by the aforementioned method are cultured in a suitable culture liquid, and then hybridomas cell strains which produce antibodies showing a specific reaction to the present antigen are selected and cloned. For cloning of antibody-producing hybridomas, in addition to the limit dilution method, the soft agar method (Eur. J. Immunol. 6: 511-519, 1976) and the like can be used. Then, the produced monoclonal antibodies are purified by methods such as column chromatography using protein A or the like.

Molecules beside the aforementioned monoclonal antibodies can also be prepared for use as a probe. For instance, regarding recombinant antibodies, a detailed description can be found in Hoogenboon's general theory and the like (Trends in Biotechnology, 15: 62-70, 1997).

In the present invention, the antigen used as probe for HCV core antibodies or the antigen used for producing the aforementioned HCV core antibodies can specifically be, for instance, a polypeptide having the amino acid sequence shown under sequence number 1 or 2, or a fused polypeptide containing multiple amino acid sequences shown under sequence numbers 3 to 6; these can be obtained through recombinant expression of DNA which encodes them.

For the detection principle here, the enzyme-labeled antibody method, fluorescent labeling method, radioisotope labeling method or other methods used in conventional immunoassays may be used; enzyme detection principles used in the enzyme-labeled antibody method include colorimetry, fluorescence, chemoluminescence, etc. Moreover, for detection of antibodies, methods generally used for antibody detection, such as the two antigen sandwich method, may be used, and likewise for detection of antigen, methods such as the one step sandwich system can be used.

One form of the present invention is a reaction system as follows. (1) A probe for HCV core antigen, for instance an anti-HCV core antigen antibody, and (2) a compound containing HCV epitopes, for instance a peptide, peptide compound or polypeptide containing an epitope of HCV polypeptide, or a mixture thereof, is fixed in solid phase to a carried used for immunoassays, for instance a microtiter plate. The carried fixed in solid phase is exposed so as to make HCV core antigen recognizable by the probe from HCV particles or particle complexes, and is reacted with the sample to be tested in a reaction buffer solution containing ingredients which do not inhibit the function of antibodies against HCV epitopes, specifically binding core antigen and antibodies against HCV epitopes contained in the sample to the carrier.

Next, after removing components in the sample which were not bound, for instance by washing the carrier with a suitable buffer solution, it is reacted with a reaction solution containing a probe which recognizes the core antigen bound to the carrier, for instance an antibody against core antigen labeled with an enzyme, and with a probe which recognizes antibodies against HCV epitopes bound to the carrier, for instance anti-human antibody mouse monoclonal antibodies labeled with an enzyme or the like, binding them specifically to core antigen and antibodies against HCV epitopes bound to the carrier. After completion of reaction, after for instance washing the carrier with a suitable buffer solution in order to remove unreacted components, the label is detected by a suitable method, allowing core antigen and antibodies against HCV epitopes contained in the sample to be detected.

Furthermore, it is self-evident to researchers in the field that this can also be applied to B/F separation methods which can generally be used for immunoassays, such as immunochromatography.

Reaction conditions suitable for antigen detection

The reaction system suitable for antigen detection in the system provided by the present invention is a system comprising conditions which are sufficiently mild so as not to cause antibodies against HCV antigen epitopes to lose their function, yet which adequately expose the region recognized by antibodies comprising the probe which recognizes HCV antigen from the HCV particles constituting complex structures within the sample.

It has been indicated that processing virus particles already separated by ultracentrifuging (Takahashi et al., 1996, J. Gen. virol., 73: 667-672) and HCV particles agglutinated and precipitated by means of polyethylene glycol, with a nonionic surfactant such as Tween 80 or Triton X100 (Kashiwakuma et al., 1996, J. Immunological methods 190: 79-89) makes detection of core antigen possible, but in the former case, the detection sensitivity is insufficient, and there are doubts as to whether the antigen is adequately exposed. Moreover, in the latter case, antibodies are deactivated through the addition of other processing agents, and the effectiveness of the surfactant itself is not addressed.

In the present invention, by first examining the conditions based on surfactants, and making the reaction solution into a composition centered on surfactants, it was made possible to efficiently detect antigens in HCV particles just by diluting the sample in the reaction solution, without having to apply pretreatment consisting of centrifuging, heating or other operations as was the case in previously reported HCV antigen detection systems.

It is necessary to provide conditions whereby core antigen can be effectively extracted from virus particles, whereby inter-reactions with various substances found in serum can be suppressed, and whereby probe and antigen can react efficiently. Effective surfactants for this purpose include surfactants having an alkyl group and a secondary to quaternary amine in the same molecule, and nonionic surfactants.

In the aforementioned surfactants having an alkyl group and a secondary to quaternary amine, the alkyl group is preferably a straight-chain alkyl group, and its carbon atom count is preferably 10 or higher, and more preferably 12 to 16. For the amine, a tertiary amine or quaternary amine (ammonium) is preferable. Specific

surfactants include dodecyl–N–sarcosine acid, dodecyl trimethyl ammonium salt, cetyl trimethyl ammonium salt, 3–(dodecyl dimethyl ammonio)–1–propane sulfonic acid, 3–(tetradecyl dimethyl ammonio)–1–propane sulfonic acid, dodecyl pyrimidium salt, cetyl pyrimidium salt, decanoyl–N–methyl glucamide (MEGA-10), dodecyl–N–betaine, etc. Dodecyl–N–sarcosine acid and dodecyl trimethyl ammonium salt are preferable.

The aforementioned nonionic surfactants preferably have hydrophilic-hydrophobic ratio between 12 and 14, with polyoxyethylene isooctyl phenyl ethers, for instance Triton X100, Triton X114, etc., or polyoxyethylene nonyl phenyl ethers, for instance Nonidet p40, Triton N101, Nikkol NP, etc., being preferable.

In the present invention, the aforementioned two types of surfactants may be used singly, but it is preferable to use them together, as concurrent use provides a greater synergistic effect.

Moreover, to detect antibodies against HCV epitopes, by reacting antigen containing HCV epitopes, a carrier to which antibodies for detecting HCV antigen are fixed in solid phase, and a sample diluted with the reaction solution provided by the present invention, and thereby efficiently detecting antigen in samples containing HCV antigen but where no HCV antibodies are present, efficiently detecting antibodies in samples where there is no HCV antigen but antibodies alone are present, and simultaneously detecting antigen and antibodies in samples where antigen and antibodies are present, it was discovered that a high signal is obtained, thus completing the present invention.

A method for simultaneously detecting viral antigen and antibodies against a viral antigen has previously been reported for HIV (Weber et al., J. Clinic. Microbiol., 36: 2235-2239, 1998). In the case of HIV, detecting the gag protein p24 as a viral antigen test is effective. On the other hand, for a test for antibodies against viral antigen, detecting antibodies against the gag protein p19 is effective. Thus, a method for simultaneously detecting viral antigen and antibodies against viral antigen is achieved by combining a method for detecting the gag protein p24 as an antigen test and a method for detecting antibodies against p19, which is a portion of gag protein, and envelope protein as an antibody test.

In this way, for cases where the epitopes of the antigen used for viral antigen detection and the epitopes recognized by antibodies in the sample used for viral antibody

detection are different, designing a method to simultaneously detect viral antigen and antibodies against viral antigen is relatively easy. The reason is that, for example in the case of HIV tests, the antigen p24 recognized by the probe used for antigen detection, for instance monoclonal antibodies against HIV epitopes, and the antigen recognized by antibodies contained in the subject's sample in the antibody tests, namely envelope protein and p19, which is a portion of gag protein, are different proteins, so the probe used for the antigen test does not ever recognize envelope protein or gag protein portion p19. Thus, decreases in sensitivity due to competitive reactions caused by binding of the probe to HIV epitopes used for antibody detection, non-specific reactions, or other mutual interference between the antigen detection system and the antibody detection system do not readily occur.

However, in the detection of antibodies against HCV epitopes, detecting antibodies against core antigen epitopes is extremely useful clinically (Chiba et al., Proc. Natl. Acad. Sci. USA 88: 4641-4645, 1991; Bresters et al., Vox Sang., 62: 213-217, 1992). Consequently, detection of antibodies against core antigen epitopes is an essential requirement for antibody detection. On the other hand, in antigen detection, since among the antigens making up the viral particle, core antigen has a lower variation rate than other antigens such as E1 and E2, detecting core antigen is the most effective method for HCV antigen detection. That is, to create an effective simultaneous HCV antigen and antibody assay, it is necessary to use the same antigen—core antigen—in the antigen detection system and the antibody detection system.

Thus, when core antigen is used without any special design, there are problems such as that monoclonal antibodies against core antigen used for antigen detection will bind to core antigen used for antibody detection and will be absorbed, lowering the antigen detection sensitivity, and HCV epitopes used for antibody tests, which bind to antigens used for antibody detection and induce non-specific reactions in the antigen test, will be masked, decreasing sensitivity.

The inventors, to resolve these problems, discovered that separating the epitopes of monoclonal antibodies used for antigen detection and the epitopes of antibodies against core antigen present in the sample makes it possible to efficiently simultaneously detect antigen and antibodies, thereby completing the present invention.

The following detailed description of embodiment examples shows examples of suitable epitope combinations for simultaneous assaying of antigen and antibodies.

Regarding epitopes of antibodies against core antigen found in samples, as a result of various epitope analyses, it was shown that the most important region is found at the N terminus of core antigen, particularly at position 1 to position 40 of the HCV polypeptide (Okamoto et al, Hepatology 15: 180-186, 1992; Sallberg et al, J. Clinical Microbiol., 30: 1989-1994, 1992; Sallsberg et al., J. Med. Virol., 43: 62-68, 1994). Furthermore, an epitope that reacts genotype-specifically is found at position 66 to 80 of the HCV polypeptide (Machida, Hepatology 16: 886-891, '92; Patent Application HEI9-209522). Thus, it is important that antigens used for detecting antibodies against HCV epitopes have the sequence of position 1 to position 40 and position 66 to position 80 of the HCV polypeptide. Consequently, for the antigen for detecting HCV antibodies, antigen CEPM containing a sequence from position 1 to position 42 and position 66 to position 80 of the HCV polypeptide is disclosed in the embodiment examples as an antigen polypeptide having a favorable sequence. CEPM is an antigen comprising an artificial sequence with the following regions of the HCV polypeptide arranged in the following order; methods for creating it are described in Patent Application HEI9-209522. Furthermore, the sequence is indicated under sequence number 10.

Order of HCV epitopes in CEPM:

(1238–1313) – (1363–1460) – (1712–1751) – (66–80) – (1686–1704) – (1716–1751) – (66–80) – (1690–1713) – (1–42)

For antigen detection, monoclonal antibodies which recognize and bind to a region with relatively few antibodies against core antigen in the sample, namely position 100 to position 130 of the HCV polypeptide, are used for the primary reaction, and for the secondary antibody recognition site for detecting core antigen bound to primary antibodies, monoclonal antibodies which recognize a region not used for antibody testing, position 40 to position 50 of the HCV polypeptide, are used to detect core antigen preserved by antibodies against position 100 to 130 of the HCV polypeptide.

Since none of these monoclonal antibodies bind to antigen containing position 1 to position 42 of the HCV polypeptide used for detecting HCV antibodies, using the aforementioned monoclonal antibodies and antigens cannot cause reaction inhibition due

to binding in the antigen detection system or the antibody detection system, making it possible to make the respective assay systems function simultaneously.

Embodiment examples

Below, the present invention is described in detail by means of embodiment examples.

Embodiment example 1. Expression and purification of expression plasmid of HCV derived polypeptide

(A) Creation of expression plasmid

An expression plasmid corresponding to the HCV core region was created by the following method. C11–C21 clone and C10–E12 clone (Unexamined Patent Publication HEI6-38765 were introduced into pUC119 to obtain plasmid pUC C11–C21 and pUC C10–E12 DNA, 1 μg of each of which was digested for 1 hour at 37°C in 20 μl restriction enzyme reaction solution (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 15 units EcoRI and 15 units ClaI enzyme) and (10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 15 units ClaI and 15 units KpnI enzyme), after which 0.8% agarose gel electrophoresis was performed and approximately 380 bp EcoRI-ClaI fragments were purified.

To a vector made by digesting these two DNA fragments and pUC119 in EcoRI and KpnI, 5 μl 10× ligase buffer solution (660 mM Tris-HCl (pH 7.5), 66 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP), 1 μl T4 ligase (350 units/μl) and water were added to bring the quantity to 50 μl, maintaining it at 16°C overnight and carrying out ligation reaction. Escherichia coli JM109 was transformed using this plasmid to obtain plasmid pUC C21–E12.

PCR was carried out on 1 ng DNA of this plasmid pUC C21–E12 using two primers (5′–GAATTCATGGGCACGAATCCTAAA–3′ (sequence number: 7) and 5′–TTAGTCCTCCAGAACCCGGAC–3′ (sequence number: 8)). PCR was carried out using the GeneAmp™ (DNA Amplification Reagent Kit, made by Perkin Elmer Cetus) kit under conditions of DNA denaturation at 95°C for 1.5 minutes, annealing at 50°C for 2 minutes, and DNA synthesis at 70°C for 3 minutes; the obtained DNA fragments were decomposed by 0.8% agarose gel electrophoresis and purified by the glass powder method (Gene Clean).

The pUC19 was digested with restriction enzyme SmaI, the DNA fragment obtained by PCR was placed into 5 μl 10× ligase buffer solution (660 mM Tris-HCl (pH 7.5), 66 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP) and 1 μl T4 ligase (350 units/μl) with water added to bring the quantity to 50 μl, and was kept overnight at 16°C to carry out ligation reaction. Escherichia coli JM109 was transformed using this plasmid to obtain plasmid pUC C21–E12 SmaI.

Digestion reaction was carried out on 1 μg of this plasmid DNA for 1 hour at 37°C in 20 μl restriction enzyme reaction solution (150 mM NaCl, 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 15 units EcoRI and 15 units BamHI enzyme), after which 0.8% agarose gel electrophoresis was performed, approximately 490 bp EcoRI-BamHI fragments were separated, and were purified by the glass powder method.

Next, 1 μg DNA of the expression vector Trp TrpE (Unexamined Patent Publication HEI5-84085) was digested for 1 hour at 37°C in 20 μl restriction enzyme reaction solution (150 mM NaCl, 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 15 units EcoRI and 15 units BamHI enzyme), 39 μl water was added to the reaction fluid, and after heat treating for 5 minutes at 70°C, 1 μl bacterial alkali phosphatase (BAP) (250 units/μl) was added, keeping it for 1 hour at 37°C.

Phenol was added to this reaction solution to carry out phenol extraction, the obtained aqueous layer was precipitated with ethanol, and the precipitate was dried. 1 μg of the obtained EcoRI-BamHI treated vector DNA and the aforementioned core 140 fragment were placed in 5 μl 10× ligase buffer solution (660 mM Tris-HCl (pH 7.5), 66 mM $MgCl_2$, 100 mM dithiothreitol, 1 mM ATP) and 1 μl T4 ligase (350 units/μl) with water added to bring the quantity to 50 μl, and were kept overnight at 16°C to carry out ligation reaction.

Escherichia coli HB101 strain was transformed using 10 μl of this reaction solution. The susceptible Escherichia coli strain used for the transformation was created by the calcium chloride method (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159-162 (1970)). Transformed Escherichia coli was applied to an LB plate (1% trypsin, 0.5% NaCl, 1.5% agar) containing 25 μg/ml ampicillin.

1.5 ml bacterial culture liquid was centrifuged to collect the bacteria, and plasmid DNA mini-preparation was performed by the alkali method (Manniatis et al., Molecular

Cloning: A Laboratory Manual (1982)). 1 μg of the obtained plasmid DNA was digested for 1 hour at 37°C in 20 μl restriction enzyme reaction solution (150 mM NaCl, 6 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 15 units EcoRI and 15 units BamHI enzyme), and agarose gel electrophoresis was carried out to select a Trp TrpE core 160 expression plasmid producing an approximately 490 bp EcoRI-BamHI fragment).

(B) Expression and purification of polypeptide encoded in clone core 160

Escherichia coli HB101 strain having expression vector Trp TrpE was inoculated onto a 2YT medium (1.6% trypsin, 1% yeast extract, 0.5% NaCl), culturing for 9 hours at 37°C. 1 ml of this culture liquid was transplanted into 100 ml M9-CA medium (0.6% Na_2HPO_4, 0.5% KH_2PO_4, 0.5% NaCl, 0.1% NH_4Cl, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.5% casamino acid, 0.2% glucose) containing 50 μg/ml ampicillin and was cultured at 37°C. When OD600 was equal to 0.3, indol acrylic acid was added to bring the final concentration to 40 mg/l, culturing for another 16 hours. This culture liquid was centrifuged to collect bacteria.

20 ml buffer solution A (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 30 mM NaCl) was added to the bacteria to make a suspension, and centrifuging was again carried out to obtain 2.6 g expression bacteria. The obtained bacteria were suspended in 10 ml buffer solution A, and after crushing Escherichia coli membranes by means of ultrasound crushing, centrifugal separation was performed to obtain an insoluble fraction containing TrpE fused polypeptide and polypeptide encoded by HCV cDNA. Buffer solution A containing 10 ml 6M urea was added to this fraction to solubilize and extract the fused polypeptide. The solubilized extract was subjected to ion exchange column chromatography using S-Sepharose to purify the fused polypeptide.

Embodiment example 2. Preparation of hybridomas

After dissolving fused polypeptide (TrpC11) prepared by the method described above in 6M urea, it was diluted in 10 mM phosphoric acid buffer solution (pH 7.3) containing 0.15 M NaCl so as to make the final concentration 1.0 mg/ml and mixed with an equal quantity of TiterMax to make a TrpC11 suspension. Said suspension, which had been prepared to make the TrpC11 concentration 0.01 to 0.05 mg/ml, was administered intraperitoneally to 4 to 6 week old BALB/c mice. Approximately 8 weeks later, the

immunized animals were injected into the tail vein with saline solution prepared to have a TrpC11 concentration of 0.005 to 0.03 mg/ml.

On the third day after the final follow-up immunization, spleens were extirpated aseptically from these immunized animals, cut into slices with scissors, broken down into individual cells using a mesh, and washed 3 times in RPMI-1640 culture medium. Mouse myeloma cell strain PAI in the logarithmic growth period which had been cultured for several days in the presence of 8–azaguanidine and from which revertants had been completely eliminated was washed in the same manner as described above, placing 1.8×10^7 of said cells and 1.0×10^8 spleen cells into a 50 ml capacity centrifuge tube and mixing. Centrifugal separation was carried out at 200× g for 5 minutes, the supernatant was removed, and 1 ml RPMI-1640 medium containing 50% polyethylene glycol (PEG) 4000 (made by Merck) was added to bring about cell fusion.

After removing PEG from the fused cells by centrifugal separation (200× g, 5 minutes), they were cultured for 1 to 2 weeks in RPMI-1640 medium containing hypoxantine, aminopterin and thymidine (hereinafter abbreviated as HAT) using a 96 well plate to cause multiplication only of hybridomas. Subsequently, they were grown in medium not containing HAT, and after approximately 2 weeks, clones producing the target antibodies were searched for using ELISA and hybridomas producing the inventive monoclonal antibodies having the desired reaction specificity were obtained.

For the obtained hybridomas, the search for strains producing the target antibodies and monocloning were carried out following the conventional limit dilution method, and the obtained hybridomas were named HC11-14, HC11-10, HC11-3 and HC11-7. Said four hybridomas were deposited with the National Research Institute of Microorganisms on 4 July 1987 as FERM BP-6006, FERM Bp-6004, FERM BP-6002 and FERM BP-6003.

Embodiment example 3. Preparation of monoclonal antibodies

Hybridomas obtained by the method described in embodiment example 2 were transplanted into the abdominal cavity of mice treated with pristane and the like, and monoclonal antibodies produced in the ascites were acquired. For purification of said monoclonal antibodies, the IgG fraction was separated by means of a sepharose column bound with protein A.

Regarding the monoclonal antibodies produced by the aforementioned 4 types of hybridomas, isotypes C11-14, C11-10, C11-7 and C11-3, it was established by means of double immunodiffusion using rabbit anti-mouse Ig isotype antibodies (made by Zymed) that C11-10 and C11-7 are IgG2a and that C11-14 and C11-3 are IgG1. The results of epitope analysis using 20 peptides synthesized with HCV core region derived sequences for the obtained 4 types of monoclonal antibodies indicated that they are monoclonal antibodies which recognize portions of the core region as shown in Table 1.

Table 1

Antibody	Recognition site
C11-14	^{41}Gly–^{50}Arg (sequence number 4)
C11-10	^{21}Asp–^{40}Arg (sequence number 3)
C11-3	^{100}Pro–^{120}Gly (sequence number 5)
C11-7	^{111}Asp–^{130}Phe (sequence number 6)

Embodiment example 4. Method for efficiently detecting antigen without pre-treatment operations

A sample containing HCV particles was diluted in reaction solution to which surfactant had been added to study the efficiency of HCV core antigen detection.

Detection of HCV core antigen was carried out by sandwich enzyme immunoassay (EIA) using monoclonal antibodies against HCV core antigen. Of the monoclonal antibodies obtained in embodiment example 3, C11-3 and C11-7 were used as antibodies to complement core antigen, and C11-10 and C11-14 were used as antibodies for detecting complemented core antigen.

EIA was basically carried out under the following conditions. Monoclonal antibodies C11-3 and C11-7 were diluted in acetic acid buffer solution to 4 μg/ml each, which solution was placed into a microtiter plate and kept overnight at 4°C. A blocking operation was carried out by washing with phosphoric acid buffer solution and adding phosphoric acid buffer solution containing 1% BSA. 100 μl reaction solution and 100 μl sample were then added and stirred and reacted for 1.5 hours at room temperature. After

removing unreacted material by washing with phosphoric acid buffer solution to which a low concentration of surfactant had been added, alkali phosphatase-labeled monoclonal antibodies C11-10 and C11-14 were added, reacting for 30 minutes at room temperature. After completion of reaction, unreacted matter was removed by washing with phosphoric acid buffer solution to which a low concentration of surfactant had been added, substrate solution (CDP-Star/emerald 11) was added, and after reacting for 15 minutes at room temperature, luminescence was measured.

Various surfactants were added to the primary reaction solution to study their effect. Using HCV antigen position serum thought to contain hardly any antibodies against HCV, with a titer of antibodies against HCV below detection sensitivity, the core antigen detection sensitivity was studied based on luminescence, and taking the luminescence for healthy human serum to be 1.0, was expressed as the reaction ratio thereto.

Table 2

				Reaction ratio of serums to healthy human serum (S/N ratio)				
				No45	No46	No3	No7	No19
	No additives			15.67	1.00	1.15	1.34	1.19
	Criterion of beneficial effect			>30.0	>2.0	>2.0	>2.0	>2.0
	Additive	HLB	%					
Anionic surfactants	Sodium dodecyl sulfate	40.0	0.5 2.0	5.42 5.73				
	Sodium dodecyl-N-sarcosinate		0.5 2.0	12.79 125.43	2.70 7.27	3.83	3.70	6.71
	Perfluoroalkyl carboxylic acid S-113 (made by ASAHI GLASS)		0.5 2.0	10.55 6.72	1.27 0.91			
Cationic surfactants	Cetyl trimethyl ammonium bromide		0.5 2.0	72.97 44.55	7.42 5.35	3.09	3.52	5.43
	Dodecyl pyridinium chloride		0.5 2.0	53.43 12.44	4.70 2.49	2.05	1.52	2.33
	n-dodecyl trimethyl ammonium		0.5 2.0	66.84 27.98	4.43 3.77	2.41	1.63	2.67
	Tetradecyl ammonium bromide		0.05	14.69				
	n-octyl trimethyl ammonium chloride		0.5 2.0	12.57 11.46		1.0	0.75	0.99
Bi-ionic surfactants	CHAPS		0.5 2.0	29.57 25.32		1.63	1.82	2.42
	Perfluoroalkyl betaine S-132 (made by ASAHI GLASS)		0.5 2.0	11.07 10.77	1.61 1.49			
	3-(dodecyl dimethyl ammonio)-1-propane sulfonic acid		0.5 2.0	57.69 113.19		4.57	3.44	5.26

Table 3

				Reaction ratio of serums to healthy human serum (S/N ratio)				
				No45	No46	No3	No7	No19
	No additives			15.67	1.00	1.15	1.34	1.19
	Criterion of beneficial effect			>30.0	>2.0	>2.0	>2.0	>2.0
	Additive	HLB	%					
Nonionic surfactants	MEGA-10		0.5	32.11	3.38			
			2.0	38.49	3.53	1.97	1.87	2.84
	Tween 20	16.7	0.5	16.88				
			2.0	12.36				
	Tween 40	15.6	0.5	14.96		1.02	0.99	1.41
			2.0	19.10		1.32	1.25	1.64
	Tween 80	15.0	0.5	12.45		1.33	1.23	1.10
			2.0	17.47				
	Nonidet P-40	13.1	0.5	43.14		3.09	2.95	4.58
	Octyl glucoside		0.5	12.48		0.90	0.60	0.97
			2.0	25.07		1.92	1.20	2.63
	Triton N101	13.4	0.5	26.50		1.85	1.62	2.70
			2.0	60.84		2.23	2.28	3.81
Other	Triton X100	13.5	0.5	27.72				
			2.0	71.08		2.90	2.34	3.86
	Triton X114	12.4	0.5	31.49		2.04	1.65	2.77
			2.0	58.62		1.92	2.11	2.51
	Triton X305	17.3	0.5	10.50		0.94	0.97	1.08
			2.0	25.91		1.30	1.24	1.87
	Triton X405	17.9	0.5	12.54		0.86	0.78	1.04
			2.0	24.92		1.21	1.24	1.25
	Benzyl dimethyl phenyl ammonium chloride		0.5	5.45	1.00			
			2.0	7.01	1.12			
	Triethylamine		0.5	3.89	0.97			
Surfactant mixtures	2% Sodium dodecyl–N–sarcosinate + 2% Triton X100			244.13		6.11	5.50	12.71

These results show that the addition of nonionic surfactants with an HLB of 12 to 14, as represented by Triton X100, increases luminescence and raises detection sensitivity for HCV antigen positive serum as compared to healthy human serum. Moreover, it was shown that the addition of surfactants simultaneously having a straight chain alkyl group and secondary to quaternary amine in their structure, as represented by sodium dodecyl–N–sarcosinate and dodecyl trimethyl ammonium, likewise improves detection sensitivity for HCV antigen positive serum. This sort of sensitivity increase was not found with the aforementioned surfactants that have an alkyl group with a carbon count of 8 or less. Furthermore, it was shown that the addition of a mixture of these two types of surfactants (in Table 2, a mixture 2% sodium dodecyl–N–sarcosinate and 2% Triton X100) further increases detection sensitivity for HCV antigen positive serum.

Embodiment example 5. Detection of core antigen in samples after HCV infection but before emergence of HCV antibodies (window period)

A commercial seroconversion panel PHV 905 (B. B. I. Inc.) was assayed as per embodiment example 4, adding 2% Triton X100 and 2% sodium dodecyl–N–sarcosinate to the reaction solution. The PHV 905 panel used here showed a shift to positive for the anti-HCV antibody test (Ortho EIA. 3.0) on the 21st day after start of observation (serum No. PHV 905-7), where the antibody titer is expressed in terms of cutoff index (S/CO) and 1.0 or greater is judged to be positive. HCV core antigen activity (luminescence) was expressed as a ratio (S/N) to luminescence of healthy human serum, which was taken to be 1.0.

As shown in Table 4, core antigen activity was observed before anti-HCV antibodies became positive, confirming that the addition of these surfactants exposes core antigenicity from virus particles, allowing them to be detected through reaction with monoclonal antibodies fixed in solid phase.

Table 4

Serum No.	Days after start of observation period	HCV core antigen activity (S/N)	Anti-HCV antibody titer (S/CO)
PHV 905-1	0	5.32	0.000
905-2	4	8.30	0.000
905-3	7	15.63	0.000
905-4	11	4.37	0.300
905-5	14	14.75	0.700
905-6	18	7.57	0.700
905-7	21	4.82	2.500
905-8	25	3.31	5.000
905-9	28	1.61	5.000

Embodiment example 6. Simultaneous detection of HCV antibodies and core antigen contained in sample

Using a sample containing antibodies against HCV epitopes but hardly any HCV antigen (human serum), it was confirmed by the following method that antibodies against HCV epitopes bind to HCV polypeptides in the primary reaction solution containing surfactant without losing activity and are detectable in the secondary reaction solution by adding anti-human antibodies, and furthermore that when core antigen is present it is possible to detect core antigen, when antibodies against HCV epitopes are present, it is possible to detect the antibodies, and when both are present, it is possible to detect both.

EIA was basically carried out under the following conditions. Recombinant antigen CEPM containing HCV epitopes was diluted in phosphoric acid buffer solution containing urea, placed in a microtiter plate, and kept overnight at 4°C. After washing with phosphoric acid buffer solution, a solution of monoclonal antibodies C11-3 and C11-7 diluted in acetic acid buffer solution was added to the plate and kept overnight at 4°C. The method for producing recombinant antigen CEPM is described in Patent Application HEI9-209522. After removing the antibody solution, a blocking operation was carried out by washing with phosphoric acid buffer solution and adding phosphoric acid buffer solution containing 1% BSA.

To this, 100 μl primary reaction buffer solution containing sodium dodecyl–N–sarcosinate and urea and 100 μl sample were added in sequence, stirred, and reacted at room temperature for 1.5 hours. After removing unreacted matter by washing with phosphoric acid buffer solution to which a low concentration of surfactant had been added, secondary reaction buffer solution containing horseradish peroxidase-labeled monoclonal antibodies C11-14 against HCV core antigen and mouse monoclonal antibodies against human IgG was added, reacting for 30 minutes at room temperature.

After completion of reaction, unreacted matter was removed by washing with phosphoric acid buffer solution to which a low concentration of surfactant had been added, and after adding substrate solution (ortho-phenylenediamine) and reacting for 20 minutes at room temperature, absorbance was measured.

Using as sample HCV antibody positive human serum confirmed to contain hardly any HCV core antigen and diluted with horse serum, it was confirmed that antibodies against HCV epitopes were detected, that reaction took place in a concentration-dependent manner, and that antibodies were detected without losing activity in the primary reaction solution.

Table 5: Simultaneous assay of HCV antigen and HCV antibodies

		(Reference example)	(Reference example)	(Present invention)
Labeled antibodies:		POD-labeled c11-14	POD-labeled anti-human IgG	POD-labeled c11-14 and POD-labeled anti-human IgG
Solid phase		c11-3 and c11-7	CEPM	c11-3 and c11-7 and CEPM
Sample				
Recombinant core antigen ng/ml	Positive serum dilution factor			
—	—	0.001	0.000	0.000
50	—	2.784	0.000	2.834
12.5	—	2.822	0.000	2.758
3.1	× 2048	1.586	0.210	1.341
0.78	× 512	0.423	0.539	0.815
0.2	× 128	0.085	1.139	1.151
0.048	× 32	0.014	1.746	1.621
—	× 8	0.000	2.161	1.824

(values are OD492/OD690)

Using recombinant core antigen added to horse serum and diluted with horse serum as the sample, assays confirmed that recombinant core antigen can be detected in a concentration-dependent manner.

In assays using as sample core antigen and human serum added in suitable quantities to horse serum, as indicated in Table 4, when recombinant core antigen alone is contained, a signal due to recombinant core antigen is obtained, when human HCV antibody positive serum alone is contained, a signal due to HCV antibodies alone is obtained, and when both are contained, a signal is obtained into which both signals are summed. Thus, it was found that the antigen detection system and antibody detection system both function without interfering with the other, allowing HCV core antigen and antibodies against HCV polypeptide epitopes to be detected.

Embodiment example 7. Method of assaying antigen and antibodies in human serum

Using healthy human samples and patients' samples, as well as serum positive conversion panel samples (BBI Inc.), simultaneous assays of antigen and antibodies were performed following the method described in embodiment example 6. For panel serum, determination results were compared with results using HCV antibody detection reagent supplied by the distributor.

The results of assays using 18 healthy samples are shown in Table 6: it was confirmed that no reaction takes place for healthy persons. Based on the distribution for healthy persons, the positive/negative discrimination value was set at 0.1.

As shown in Table 7, HCV positive samples all yielded positive values.

On the other hand, as shown in Table 8, positive determinations were obtained for points 1 through 6, where positives could not be discerned in antibody tests with panel serum. These points, for which the Amplicor HCV test gave a positive determination, correspond to the so-called window period, confirming that samples in the window period also give a positive determination.

Table 6

Sample number		Absorbance
Healthy person	1	0.063
	2	0.057
	3	0.066
	4	0.025
	5	0.045
	6	0.063
	7	0.047
	10	0.033
	11	0.036
	13	0.037
	14	0.030
	15	0.028
	16	0.031
	17	0.040
	18	0.051
	19	0.052
	20	0.031
	21	0.053
Mean		0.044

Table 7

Patient sample	Absorbance
3	2.892 positive
16	2.335 positive
45	0.394 positive
84	2.769 positive

Table 8

Panel serum	Absorbance	Determination	Antibody assay	Amplicor HCV test
PHV907-1	0.557	Positive	Negative	Positive
2	0.397	Positive	Negative	Positive
3	0.357	Positive	Negative	Positive
4	0.224	Positive	Negative	Positive
5	0.192	Positive	Negative	Positive
6	0.247	Positive	Positive	Positive
7	2.414	Positive	Positive	Positive

Embodiment example 8. Preparation of monoclonal antibodies

New hybridomas were prepared by the method described in embodiment example 3, which were named HC11-15. They were deposited with the National Research Institute of Microorganisms on 16 July 1999 as FEAM BP-9782. Purifying the monoclonal antibodies produced by these hybridomas and assaying for isotype showed them to be IgG1. Based on the results of epitope analysis using 20 peptides synthesized with sequences of the core region, these monoclonal antibodies were found to specifically recognize ^{15}Thr-^{30}Ile (sequence number 9).

Embodiment example 9. Antibody titer assay for monoclonal antibodies

Recombinant core antigen (Trp c11) was diluted in 10 mM phosphoric acid buffer solution (pH 7.3) containing 6M urea to bring the final concentration to 2 μg/ml, and 100 μl was placed in each well of a microplate. After letting it rest overnight at 4°C, it was suctioned and washed twice with 10 mM phosphoric acid buffer solution (pH 7.3). After adding 350 μl 10 mM phosphoric acid buffer solution (pH 7.3) containing 0.5% casein to

each well and incubating for 1 hour at room temperature, the wells were suctioned. Various monoclonal antibodies (C11-3, C11-7, C11-10, C11-14 or C11-15) diluted sequentially with reaction solution were added to the wells and reacted for 1 hour. After washing, peroxidase-labeled mouse antibodies were added, reacted for 30 minutes, and washed, after which substrate solution containing ortho-phenylenediamine and hydrogen peroxide was added to carry out enzyme reaction. After reacting for 30 minutes at room temperature, 2N sulfuric acid was added to stop the enzyme reaction and absorbance at 492 nm was determined with a microplate reader. The results are shown in Figure 1.

C11-15 had the highest antibody titer, and when used as secondary antibody, showed that detection with high sensitivity is possible.

Embodiment example 10. Sandwich ELISA assay based on differences in monoclonal antibodies fixed in solid phase

Various monoclonal antibodies (C11-3, C11-5 and C11-15; C11-3 and C11-7; C11-3 and C11-15; C11-3 alone; C11-7 alone; or C11-15 alone) were diluted in 10 mM phosphoric acid buffer solution (pH 7.3) to bring the final concentration to 6 μg/ml, and 100 μl was added to each well of a microplate. After letting it rest overnight at 4°C, it was suctioned and washed twice with 10 mM phosphoric acid buffer solution (pH 7.3). 350 μl 10 mM phosphoric acid buffer solution (pH 7.3) containing 0.5% casein was added to each well, and after incubating for 2 hours at room temperature, the wells were suctioned. 100 μl HCV-RNA positive, anti-HCV antibody negative sample and 100 μl reaction solution were added to each well and allowed to react for 1 hour at room temperature. After washing, peroxidase-labeled anti-core antigen monoclonal antibodies (a mixture of C11-14 and C11-10) were added, reacted for 30 minutes and washed, after which substrate solution containing ortho-phenylenediamine and hydrogen peroxide was added to carry out enzyme reaction. After reacting for 30 minutes at room temperature, 2N sulfuric acid was added to stop the enzyme reaction, and absorbance at 492 nm was determined with a microplate reader. The results are shown in Figure 2.

While detection sensitivity was fairly low with C11-15 alone fixed in solid phase, when C11-15 was mixed with C11-3 or C11-7 and fixed in solid phase, the detection sensitivity was shown to increase.

Embodiment example 11. Expression and purification of epitope chimera antigen

Escherichia coli transformant CEPM/HB101 strain was cultured overnight at 37°C in LB medium containing 100 μg/ml ampicillin. This was inoculated into M9-CA containing 100 μg/ml ampicillin at 1% concentration and cultured overnight at 37°C. After completion of culturing, bacteria were collected by centrifuging, resuspended in 50 ml lysis solution (50 mM Tris-HCl (pH 8.5), 30 mM NaCl, 5 mM EDTA), and 1 ml lysozyme solution (10 mg/ml lysozyme) was added thereto, treating for 1 hour at 37°C. Cells were disintegrated by subjecting this suspension to ultrasound treatment (twice at 150 W for 90 seconds). The insoluble fraction was recovered by centrifuging for 30 minutes at 15000 rpm at 4°C. The insoluble fraction was resuspended in 50 ml solution A (50 mM Tris-HCl (pH 8.5)) containing 1% NP40 and homogenized (5 strokes at 1500 rpm). The suspension was centrifuged for 30 minutes at 15000 rpm at 4°C to recover the insoluble fraction. The insoluble fraction was resuspended in 50 ml solution A containing 6M urea and homogenized (5 strokes at 1500 rpm). The soluble fraction was recovered by centrifuging the suspension for 30 minutes at 15000 rpm at 4°C.

Epitope chimera antigen was purified from antigen solution solubilized with a solution containing 6M urea by means of ion exchange using S-sepharose HP column (Pharmacia) and gel filtration using Superdex 75pg (Pharmacia).

The base sequence of the DNA encoding the above chimera antigen is shown under sequence number: 10, and the amino acid sequence of the chimera antigen is shown under sequence number: 11.

References to deposited biological material and depositary institution as per Rule 13bis of the Patent Cooperation Treaty

Depositary institution	Name:	Agency of Industrial Science and Technology, National Institute of Bioscience and Human Technology
	Address:	1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan
Biological material (1)	Designation:	HC11-3
	Accession number:	FERM BP-6002
	Date of deposit:	4 July 1997

(2) Designation: HC11-7
Accession number: FERM BP-6003
Date of deposit: 4 July 1997

(3) Designation: HC11-10
Accession number: FERM BP-6004
Date of deposit: 4 July 1997

(4) Designation: HC11-11
Accession number: FERM BP-6005
Date of deposit: 4 July 1997

(5) Designation: HC11-4
Accession number: FERM BP-6006
Date of deposit: 4 July 1997

(6) Designation: HC11-15
Accession number: FERM BP-6782
Date of deposit: 16 July 1999

Claims

1. A method for assaying hepatitis C virus (HCV) distinguished in that HCV core antigen is assayed by means of its binding to a probe in the presence of a surfactant having an alkyl group with a carbon atom count of 10 or higher and a secondary to quaternary amine and/or a nonionic surfactant.

2. A method as described in claim 1, wherein the aforementioned surfactant having an alkyl group and a secondary to quaternary amine is a surfactant having an alkyl group with a carbon atom count of 12 to 16 and a tertiary or quaternary amine.

3. A method as described in claim 1 or 2, wherein the aforementioned tertiary or quaternary amine surfactant is dodecyl–N–sarcosine acid, cetyl or dodecyl trimethyl ammonium salt, 3–(dodecyl dimethyl ammonio)–1–propane sulfonic acid, dodecyl pyrimidium salt, or decanoyl–N–methyl glucamide (MEGA-10).

4. A method as described in any of claims 1 through 3, wherein the aforementioned nonionic surfactant is a surfactant having a hydrophilic-hydrophobic ratio (HLB) of 12 to 14.

5. A method as described in any of claims 1 through 4, wherein the aforementioned nonionic surfactant is a polyoxyethylene isooctyl phenyl ether or polyoxyethylene nonyl phenyl ether.

6. A method for assaying hepatitis C virus, distinguished in that anti-HCV antibodies are assayed by means of their binding to a probe, along with assaying HCV core antigen by the method described in claims 1 through 5.

7. A method as described in claim 6, wherein the aforementioned probe for anti-HCV antibodies is an HCV-related polypeptide.

Fig. 1

Monoclonal antibodies Antibody titer